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ORIGINAL PAPER

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Identification of candidate genes for in vitro androgenesis induction in maize

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Abstract Extensive studies have been conducted to understand the genetic control of in vitro androgenesis, but little is know about the genes and the mechanisms involved in the switch that allows an immature pollen grain to develop as an embryo. We have developed two maize isogenic lines with high androgenetic aptitude, named AH5-44 and AH5-49, through backcross and selection from a high-responsive DH229 line on the non-responding A188 line genetic background. The genomic structure of these two lines was precisely described with microsatellite markers. Five regions retained from the parent DH229 highly responsive to androgenesis were localised in both AH5-44 and AH5-49. Sequences expressed on microspores extracted from the four lines were amplified using a cDNA-AFLP protocol. For each line, eight culture conditions were compared: microspores extracted after tassel recovery, after 7 or 14 days in cold room and after 1–4 days of in vitro culture. This genetic and developmental screening allowed us to identify four sequences, including a new *HSP70*-like candidate gene. Possible implication of the identified sequences in androgenesis response is discussed.

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Correspondence and request for material (near isogenic line seeds and pre-amplified cDNA-AFLP samples are available for other groups to identify new sequences potentially involved in androgenesis using a larger set of primer combinations on cDNA-AFLP samples) should be addressed to P.B. (e-mail: barret@clermont.inra.fr)
Sequence data from this article have been deposited in the EMBL/GenBank data libraries under accession nos. AY222837 (HSP70-like), CD766057 (E9 M1-130), CD766058 (E13 M1-550), and CD766059 (E9 M5-320)

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Introduction

Maize lines selected for high aptitude to in vitro androgenesis (Petolino and Jones 1986; Dieu and Beckert 1986) can be considered as performing tools to study and dissect functions of genes involved in the switch between normal gametophytic pollen development and direct sporophytic embryo development (Cordewener et al. 1996). This switch, which must be effective before the asymmetric division of the pollen cell, might be amplified by different abiotic stress, like heat in rapeseed (Lichter 1982) and cold in corn (Brettel et al. 1981), and might be related to mechanisms described in somatic embryogenesis (Hecht et al. 2001). Studying the genes and the mechanisms involved in the switch of microspore embryogenesis mediated by stress can also provide information on pollen development (for review, see Twell 2002) and a means of managing more efficiently doubled haploid production for breeding purposes (for review, see Wang et al. 2000).

The search for genes involved in the response to androgenesis has been approached with molecular mapping and QTL analysis (Cowen et al. 1992; Murigneux et al. 1994; Beaumont et al. 1995; Manninen 2000). These authors concluded that genetic control of androgenesis induction in specific material might be oligogenic. Another strategy could be based on the candidate gene approach (for review, see Pflieger et al. 2000). In both ways, identified candidate genes can be tested for putative implication in the biological mechanism by genetic linkage analysis with one QTL involved in the expression of the character. Vergne et al. (1993) have identified a sporophytic protein that could be involved in androgenesis in maize. Cordewener et al. (1995) have shown that HSP68 and HSP70 might be involved in the induction of embryogenesis in *Brassica napus* microspores. Beaumont et al. (1995) suggested that ABA or GA3 could be related

to androgenetic aptitude as five *vp* (viviparous) genes were mapped near some QTLs for androgenesis. The involvement of ABA in androgenesis has also been suggested for *Hordeum vulgare* (Wang et al. 1999). Genes differentially expressed between induced microspores and pro-embryos have been identified by Magnard et al. (2000). Despite these efforts, the biological mechanisms involved in plant androgenesis induction are still poorly described (for review, see Touraev et al. 1997).

In order to approach the molecular mechanisms underlying androgenesis induction, we chose in the present study a direct transcriptional approach using cDNA-AFLP, comparing near isogenic lines (NILs) developed in a narrow genetic base between contrasted genotypes. First, the NILs were checked for good isogenic structure, using microsatellite markers. This technique allowed us to clearly identify the five genomic sectors of a high-responsive line retained by selection for a non-responsive background of the two NILs. Second, four sequences presenting different genetic and/or developmental profiles were identified using the cDNA-AFLP protocol. One of these sequences presented a major expression after microspore culture in A188 and in the lowest responsive NIL. It corresponds to a new maize *HSP70*-like gene. The possible role of these sequences in the androgenesis process is discussed.

Materials and methods

Plant materials

A188 is a well-known maize line extensively used for molecular and cellular genetic studies, without any detectable androgenetic response (Brettell et al. 1981). DH5, DH7 and DH99 are doubled haploid lines with the high androgenetic response (Barloy et al. 1989). DH229 is a doubled haploid line obtained from the cross between the two unrelated DH lines DH5 and DH7 (Barloy et al. 1989). PK6 is a maize line developed in our laboratory, with high aptitudes to in situ gynogenetic induction. AH5-44 and AH5-49 are the two near isogenic lines with high androgenetic response described in this paper. A188, DH229, AH5-44 and AH5-49 plants were grown together as described by Gaillard et al. (1991).

Anther culture and androgenesis response estimation

Anther culture in solid medium was such as described by Dieu and Beckert (1986). The anther culturability (AC) was defined as the ratio of the number of embryogenic anthers to the total number of plated anthers, as described by Murigneux et al. (1994).

Microsatellite amplification and electrophoresis on 5% agarose gels

Microsatellite markers were chosen in the maize database (MaizeDB, http://www.agron.missouri.edu/), and PCR amplification was conducted as described on the Web site. Electrophoresis was conducted on 5% agarose gels (Eurobio, Les Ulis, France) on 0.5× TAE buffer (Sambrook and Russell 2001) at 5 V/cm and 100 mA for 4–5 h, depending of the size of the band. Gels were soaked in a 0.5 μg/ml ethydium bromide solution, visualized under UV light, and the picture was captured with a CCD camera.

Microspore isolation and in vitro culture conditions

Microspore isolation and culture were as described in Gaillard et al. (1991).

Microspore isolation, mRNA extraction and cDNA AFLP amplification

Differential expression of the genes was studied in microspores, using a cDNA-AFLP protocol. A first screen was made by comparing the expressed profiles of microspores extracted just after tassel collection (T0), following 7 (T7) or 14 (T14) days at low temperatures (the sample recovered after 14 days on the AH5-44 line was discarded because of contaminations). This first part of the screen made it possible to look for genes expressed in response to cold and genes that could be involved in future aptitudes of microspores to androgenesis.

To search for genes expressed after cold treatment, we placed the microspores in liquid medium and extracted RNA from these microspores after 1 (T7+1), 2 (T7+2), 3 (T7+3) and 4 (T7+4) days of culture (samples cultured for 1 and 2 days for AH5-44 were discarded because of contaminations).

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen). cDNA-AFLP protocol was as described in Bachem et al. (1998), except that *Eco*RI and *Tru*9I (isoschizomer of *Mse*I) restriction endonucleases were used with the same primers as in classical AFLP, except for a double-base extension for selective amplification (39 combinations tested, available upon request). DNA fragments were separated by standard 5% denaturing polyacrylamide gel electrophoresis and stained with silver nitrate as described in Bassam et al. (1991). Bands of interest were recovered by direct cutting on the gel and, DNA was eluted overnight in 50 μl ultrapure water. DNA fragments were also re-amplified with *Eco*+0 and *Mse*+0 primers.

HSP70-like PCR amplification

PCR-specific primers were designed using Oligo 4 software. The sequences of the five oligonucleotides employed were H1UP 5′[GCTGTTGCCTACGGAGC-TAC]; H1LP 5′[ATTCCAGTCGACACTGAAGC]3′; H2UP 5′[GGGCTCTCAGGAGGCTAAGG]3′; H3UP 5′[TGGTCTTATCGACTGACTCC]3′ and H2LP 5′[TCAATTTACAAGCCAACCAC]3′. The PCR reaction mixture (25 µl) contained 1× *Taq* polymerase buffer (Qiagen) containing 1.5 mM $MgCl_2$, 150 µM of each dNTP, 10 pmole of each primer, 1 U *Taq* polymerase (Qiagen) and 50 ng plant genomic DNA. PCR was performed in an MJ Research PCR apparatus with one cycle of 94°C/2 min, 35 cycles of 94°C/45 s, 55°C/45 s, 72°C/2 min and a final extension step of 72°C/5 min. PCR products were resolved on standard 2% agarose gel.

Genetic mapping

Genetic mapping was performed on an F_2 population originating from the cross DH99 (a doubled haploid line with high androgenetic capacities) and a W23-related line. The *HSP70* locus was mapped on a frame built from 100 microsatellite loci (one per bin) and 64 F_2 segregating individuals. Linkage analysis was performed using Join-Map software with standard parameters.

Southern blots

DNA was extracted according to the method of Dellaporta et al. (1983). *HSP70* PCR probe purification, labelling and Southern hybridization were performed as described in Barret et al. (1998).

Sequencing reactions and sequence software analysis

Sequencing reactions were made by Genome Express (Meylan, France) or handled with silver sequence kit (PROMEGA), following the manufacturers' instructions. Electrophoresis of the sequencing products and coloration were performed on the same gels as cDNA-AFLP, except for a 6% acrylamide concentration. Because of the proximity of another AFLP fragment, E9-M3 530-bp fragment recovery, reamplification and sequencing were made twice, and the same sequence was obtained. Sequence analysis was made using the NCBI Web site (http://www.ncbi.nlm.nih.gov/) with BLAST software (Altschul et al. 1997). BLAST analysis was conducted on non-redundant, EST (other than human and mouse) and GSS (genome survey sequences) banks and TblastX analysis on the Swissprot bank (last analysis made on 11 June 2004). Sequences were compared with LAGIGN software (Huang and Miller 1991) or with Clustal W [Thompson et al. (1994) on the INFOBIOGEN Web site, http://www.infobiogen.fr].

Results

Androgenetic induction capacities can be transferred through selection on isogenic lines

The two isogenic lines AH5-44 and AH5-49 were recovered after three backcrosses and two generations of selfing with anther culture and double haploid production after each backcross and selection for the plant expressing the highest androgenesis aptitude among 100 tested per generation. After this selection, AC was 40% for AH5-44 and 50% for AH5-49. Complete androgenesis data of the eight lines used in this work are presented in Table 1.

Isogenic lines differ for a small number of DH229 introgressed chromosome segments

At first, the presence of DH229 introgressed fragments in the A188 background was checked using SSRs. This work allowed us to localise on the maize genetic map the introgressed fragments of DH229 potentially involved in androgenesis. To obtain a good coverage of the genome, one polymorphic microsatellite was identified in each bin of the maize genetic map (Sharopova et al. 2002), except for five bins on chromosomes 1, 3 and 9. As expected, a majority of the microsatellites presented A188 alleles (Figs. 1a, 2). Some of microsatellites allowed us to identify five DH229 regions on chromosomes 1, 2, 3 and 8 (Fig. 1b, for example, on 8.08/09 bins; Fig. 2). The majority of these regions were homozygous, but two regions in AH5-49 presented a residual heterozygosity (Fig. 2, in bins 2.09 and 8.03–8.04; Fig. 1c, for example, on 8.03 bin) and one region in AH5-44 (in position 1.09, Fig. 2). Surprisingly, we identified two positions (in 2.07 and in 8.09) with non-parental allelic form. With this strategy, we obtained a maize genome coverage of 95% (95/100 bins), corresponding to 111 polymorphic markers

Table 1 Androgenesis response of lines A188 (genetic background), DH229 (donor plant with high androgenesis response), AH5-44 and AH5-49 (isogenic lines), PK6 (non-responsive line), DH5, DH7 and DH99 (doubled haploid lines with high androgenesis response). *AC* Anther culturability, i.e. percentage of anther developing at least one embryo, *Emb* embryo for 100 plated anthers (androgenetic developed embryo detectable at the macroscopic level), *Pl* plants for 100 plated anthers. For each genotype, 2,000–3,000 anthers were plated

	AC	Emb	Pl
A188	0	0	0
PK6	0	0	0
DH229	71	350	34
AH5-44	40	88	16
AH5-49	50	110	15
DH5	37	31	1
DH7	28	36	1
DH99	43	29	3

checked (Fig. 2). The percentage of DH229 genome introgressed in AH5-44 and AH5-49 was estimated to be 3.7% (7 DH229 haploid bins/190 haploid bins tested) and 5.2% (10/190), respectively. As the high level of isogenicity of the NILs was checked, these lines could be used for differential screening purposes.

Four sequences that are differentially expressed during the stress-induced switch of microspore embryogenesis were detected

Our goal was to identify genes involved in the stress-induced switch of microspore embryogenesis. For this, we used cDNA-AFLP technology. Considering the various steps involved in the whole androgenesis response, we have supposed that our screen could allow us to identify genes differentially expressed in response to cold, to in vitro culture, to microspore division and development, which are in their majority not directly involved in androgenesis. To avoid this, the cDNA-AFLP protocol was applied to the two parental lines A188 and DH229 and to the isogenic lines AH5-44 and AH5-49 previously described, and only the polymorphic bands with a DH229 allelic form found in AH5-44 and/or AH5-49 were selected.

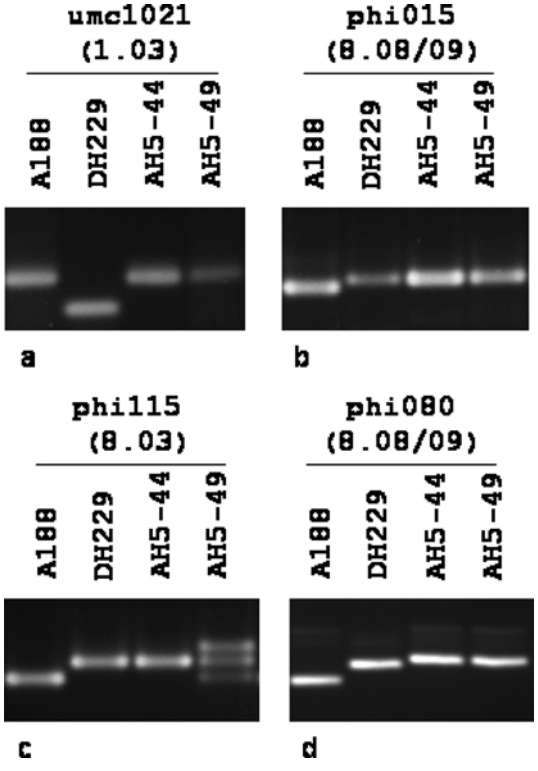

Fig. 1 Characterisation of high androgenesis aptitudes of AH5-44 and AH5-49 isogenic lines, with microsatellite markers. *A188* Genetic background, *DH229* donor parent for androgenesis aptitudes. The four different microsatellite types of profiles obtained are presented: **a** A188 allelic form for the two isogenic lines; **b** DH229 allelic form for the two isogenic lines; **c** DH229 allelic form for the AH5-44 and heterozygous form for AH5-49 (two bands issued from the two parents and one third corresponding to a heteroduplex between the other two); and **d** non-parental allelic form for the two isogenic lines. Amplified DNA fragments (about 200 bp) were separated by 5% agarose gel electrophoresis

Four profiles corresponding to the defined screen were detected with the 39 primer combinations used (Fig. 3)

E9 M3-530 fragment presented a globally higher expression rate during the culture phase and was only expressed in A188 and AH44. BlastN searches in EST databases gave a perfect match with *Zea mays* BG837965 EST (Fig. 4). BlastX analysis of BG837965 in the SwissProt database showed significant alignments with HSP70 proteins from a wide range of organisms and showed a significant alignment (*E*-value = $3e^{-86}$) with a pfam00012 HSP70 conserved domain. The best score on Swissprot (Viridiplantae) was obtained for a *Lycopersicon esculentum* HSP70 (P27322, *E*-value = e^{-103}, 70% identity on 262 amino acids). The best score for a maize protein was obtained for an HSP70 described by Rochester et al. (1986) (P11143, *E*-value = $2e^{-96}$, 68% identity on 262 amino acids). BG837965 gave also a match with CG228472 GSS (*E*-value = e^{-129}). CG228472 showed a significant alignment with P11143 (*E*-value = $5e^{-79}$, 75% identity on 188 amino acids). Alignment of E9 M3-530 (AY222837), BG837965 and CG228472 DNA sequences and of P11143 protein sequence is shown in Fig. 4. No alignment could be obtained for the end of the BG837965 sequence (from position 782 to position 992). Taken as an individual sequence, this region showed no significant alignments on the databases with BlastX on Swissprot or with TblastX on non-redundant, translated GenBank sequences. PCR amplification of cDNA samples with six combinations of specific primers (H1, H2 and H3UP in combination with H1 and H2LP) showed a band only in A188 and AH44 samples (data not shown). PCR amplification of genomic DNA samples of A188, DH229, AH44, AH49, DH5, DH7, DH99 and PK6 lines with the same primers showed the same bands only in A188, AH44 and PK6 samples (data not shown). Genetic mapping of the band obtained with primers H3UP + H1LP on an F_2 population (see 'Materials and methods' for details) showed that the *HSP70*-like locus was closely linked to the *umc1335* at position 390.2 cM at bins 1.06/1.07 (SSR IBM map). Southern blot with the *HSP70*-like fragment showed one specific band for A188 and AH49 samples and one specific band for DH229 and AH44 samples (Fig. 5).

Expression of the E9 M1-130 fragment could only be detected in DH229 and AH5-44 lines and only after 2 days of culture (T7+3 and T7+4, Fig. 3). This particular sequence showed no homology with sequences from the databases of non-redundant sequences, EST sequences and the complete rice genome sequence.

Fragment E13 M1-550 showed constitutive expression in the conditions tested, but was only expressed in the high-responsive line DH229 and in the highest responsive NIL, AH5-49. The sequence obtained showed high

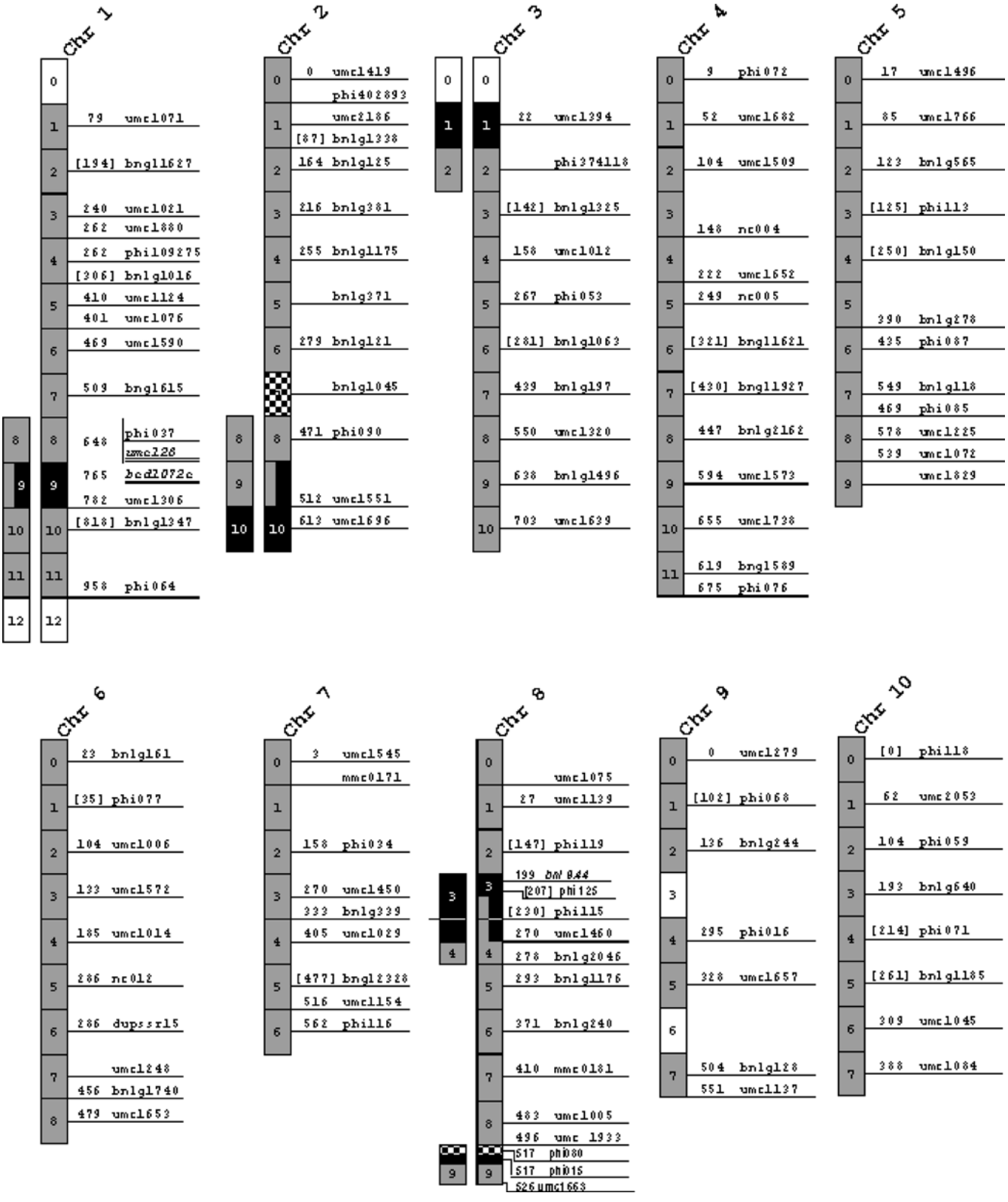

Fig. 2 Schematic map of the genome of the two isogenic lines AH5-44 and AH5-49. For each bin, at least one microsatellite was checked for allelism of A188 (*grey boxes*), DH229 (*black boxes*), non-parental allelic form (*dark grey boxes*) or was not checked if no polymorphism was available for (a) tested microsatellite(s) (*white boxes*). Two *vertical black andgrey bars* were drawn when an A188–DH229 heterozygous segment was detected. For each marker, position in cM from the bottom of the linkage group was indicated when it was available on IBM neighbours, verson 21, map or when it was possible to extrapolate it from other maps (number under *brackets*). Loci *umc128*, *bcd1072c* and *bnl9.44* (*boldface*) were not directly mapped on our NILs but were put on the scheme in regard to their position on IBM Neighbours, version 21, map because of their particular interest. Complete schematic map for AH5-49 (on the right of each chromosome) and partial map of introgressions for AH5-44 are shown

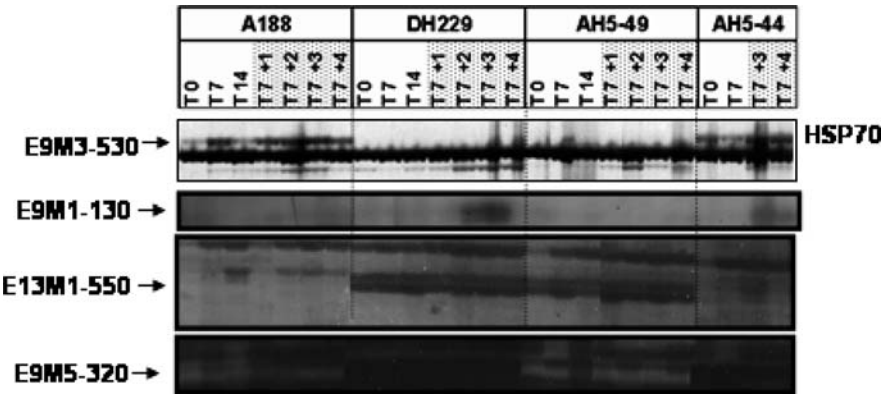

Fig. 3 Genetic and developmental cDNA-AFLP differential amplification. A genetic screen was conducted between A188 (genetic background), DH229 (donor parent for high androgenesis aptitudes) and the two isogenic androgenetic lines AH5-44 [40% anther culturability (AC)] and AH5-49 (50% AC). The developmental screen allowed us to analyse cold conditions (*T0* extracted microspores without cold treatment; *T7, T14* after 7 and 14 days of cold treatment, respectively) and in vitro culture conditions (*T7 +1*, *+2*, *+3* and *+4*, corresponding to 7 days under cold conditions and 1, 2, 3 and 4 days of culture, respectively). E9, M6 and M3 were the AFLP primers used. The estimated sizes of the bands of interest in base pairs were 250, 300 and 530. AFLP fragments were resolved on 4.5% acrylamide under denaturing conditions and revealed with $AgNO_3$

homology with only one EST (AI901465, *E*-value = $3e^{-42}$), but with no significant match with any gene of known function.

Fragment E9 M5-320 showed strong constitutive expression in the conditions tested, but only in DH229 and AH5-44 NIL. The sequence obtained showed high homology with an aspartic proteinase prophytepsin (P42210, *E*-value = $3e^{-33}$) from *H. vulgare*, described in Kervinen et al. (1999).

Discussion

Five regions were identified in the recombinant lines AH5-44 and AH5-49, corresponding to introgressed fragments of DH229 (in bins 1.09, 2.10, 3.00–3.01, 8.03–8.04 and 8.08 for the two lines and bin 2.09 specifically for AH5-49). As the *HSP70*-like gene was not mapped on a previously detected introgressed region, we can expect that all the DH229 introgressed fragments were not detected with the microsatellite frame used, and that more microsatellites should be used to obtain an better genomic description of the NILs. Among the segments

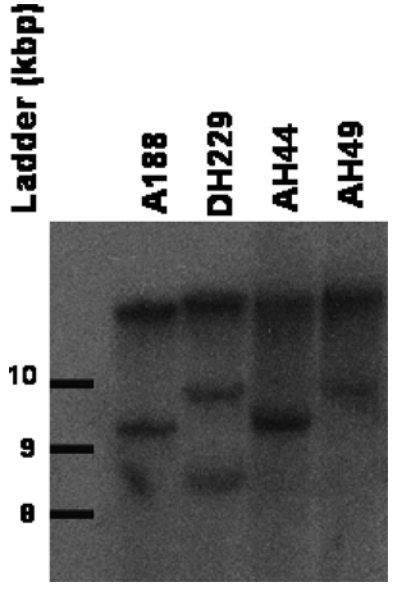

Fig. 5 Southern blot hybridized with an *HSP70*-like specific probe (see Fig. 4). DNA from A188 (non-responsive line), DH229 (parental responsive line), AH44 and AH49 (near isogenic line responsive lines) was digested with *Hin*dIII, and generated fragments were separated by electrophoresis on 0.8% agarose gel

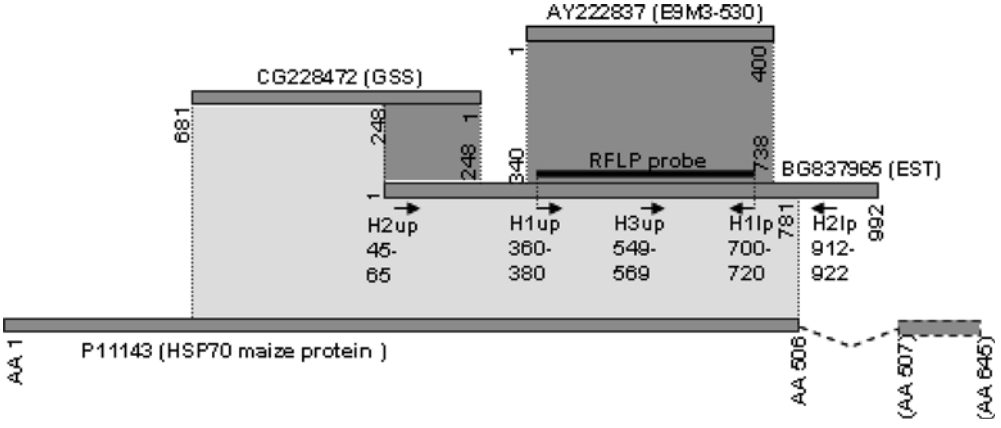

Fig. 4 Alignment of E9 M3-530 (AY222837), BG837965 and CG228472 DNA sequences and of P11143 protein sequence. Positions are indicated in base pairs (DNA sequences) and in amino acids number (protein). *Dark grey areas* DNA sequence homology >98%, *light grey areas* protein sequence homology between 68% and 75%. Position of the RFLP probe used in Fig. 5 is indicated. See 'Materials and methods' for sequences of the primers

detected, the region 8.03–8.04 around the marker *umc9.44* (Fig. 2) was previously identified by QTL analysis for AC, using population R6 × DH99 (Murigneux et al. 1994). Beaumont et al. (1995) have also localised a similar QTL on the same region. The difference in the anther culturability rate between AH5-44 and AH5-49 was 10% (Table 1). Three differences in the genome structure of AH5-44 and AH5-49 could explain the AC rate difference between these two genotypes. First, two segments localised on 2.09 and 8.03–8.04 were partially heterozygous for A188-DH229 in AH5-49 and homozygous for DH229 in AH5-44. These segments could contain a dominant or partially dominant allele for DH229 for genes involved in androgenetic response. According to Fu and Dooner (2002), improvement of the fitness of the plants could also result from one androgenetic gene specifically present in DH229. A third segment localised in 1.09 was homozygous for DH229 allele in AH5-49 and heterozygous in AH5-44. This segment could contain the DH229 allele of genes involved in androgenesis response with an additive effect. Backcrossing of isogenic lines with A188, selfing, and identification of lines modified in only one point with microsatellite-assisted selection should help us to determine the importance of each segment in androgenesis. The percentages of introgressed bins identified with microsatellite markers in AH5-44 and AH5-49 were 3.7% and 5.2%, respectively. This indicates that most of the DH229 genome not linked to regions involved in androgenesis was eliminated from NILs.

The E9 M3-530 fragment had significant alignments with HSP70 from a wide range of organisms and showed a significant alignment with the HSP70 conserved domain. These results showed that the gene herein identified is a member of the HSP70 family. As a perfect match was obtained with the EST database but with none of the protein databases, we can conclude that we identified a new member of the HSP70 family in *Z. mays*. Members of this family have been well described in maize reproductive tissues (Dupuis and Dumas 1990), and several studies have particularly focused on HSP70 (Bates et al. 1994; Gagliardi et al. 1995). The possible implication of HSP70 in embryogenesis of *B. napus* microspores has been suggested by Cordewener et al. (1994 and 1995), who suggested that induced synthesis and nuclear transfer of HSP70 might be involved in inappropriate regulation of the cell-cycle timing, hence influencing the switch in developmental fate from pollen development to embryogenic development. In our experiments, the HSP70 detected seemed to have a negative effect on androgenesis as its expression was only observed in the non-responsive line A188 and in the lowest responsive line AH5-44. Lee et al. (1996) have suggested, using HSP70-antisense *Arabidopsis thaliana* plants, that HSP70 could act as a negative regulator of HSF (heat shock transcription factors) in plants. HSF are important regulators of cellular functions in eukaryotes that activate transcription of heat shock genes (for review, see Schöffl et al. 1998). At least three HSF genes are expressed in maize (Gagliardi et al. 1995), and six are annotated in the public EST database (Gai et al. 2000). Interactions between HSF factors and HSP70 protein have been demonstrated for HSF1 from HeLa cells (Shi et al. 1998) and for HSF1 from *A. thaliana* (Kim and Schôffl 2002). Therefor, we suggest that expression of the *HSP70* gene described could have a negative feedback on a HSF important for the androgenesis switch in low responsive lines. This HSF could have functions related to LEC1, a transcription factor homologue sufficient to induce embryo development in vegetative cells (Lotan et al. 1998). As the polymorphism observed between high responsive lines (DH229 and AH5-49) and low responsive ones (A188 and AH5-44) was also retrieved by PCR analysis at the DNA level, we can conclude that this polymorphism was not an expression polymorphism but a DNA sequence polymorphism. Further analysis with different PCR primers led to the same results. This indicates that the DH229 *HSP70*-like allele was absent or very different from the A188 allele. Southern blot analysis showed that one specific band (9.2 kb, Fig. 5) was present in A188 and AH44 lines, corresponding probably to the specific band detected by PCR. This analysis also showed that two related sequences (bands at 8.3 kb and 9.7 kb, Fig. 5) were present in the DH229 line, and that one related sequence (9.7 kb) was present in the AH49 line. RFLP analysis of a segregating population from a cross A188 × DH229 could indicate if the allele in DH229 corresponding to the 9.2 kb band in A188 was the 9.7-kb band or a null allele. The *HSP70*-like gene was localised in bins 1.06–1.07 (closely linked to microsatellite *umc1335*) on our microsatellite map. A QTL for androgenesis has already been localised around the probe *BNL7.08* in bin 1.07, using the cross A188 × HD7 (Murigneux et al. 1994), providing a possible functional validation of our observations. In conclusion, we describe a new *HSP70*-like gene in *Z. mays*. A specific allelic form found in the A188 line could explain the 10% difference between AH5-44 and AH5-49. As this gene was genetically linked to a QTL previously described, it could be one of the genes involved in androgenesis in maize.

Expression of the E9 M1-130 fragment could only be detected in the DH229 and AH5-44 lines and only after two days of culture (T7+3 and T7+4, Fig. 3). This particular sequence had no homology with sequences from the databases (non-redundant sequences, EST sequences and rice complete genome sequence). This can be the consequence of the small size of the sequence obtained (74 bp). The corresponding gene can also have a very short window of expression and thus be under-represented in the cDNA banks used for EST sequencing programmes. This particular fragment was expressed in DH229 and in the NIL AH5-44 and could have been involved in androgenetic response.

Fragment E13 M1-550 showed constitutive expression in the conditions tested, but was only expressed in the high-responsive line DH229 and in the highest responsive NIL, AH5-49. The sequence obtained showed high homology with only one EST (AI901465, *E*-value =

$3e^{-42}$), but with no significant match with a gene of known function.

Fragment E9 M5-320 showed strong constitutive expression in the conditions tested, but only in DH229 and AH5-44 NIL. The sequence obtained presented high homology with an aspartic proteinase prophytepsin (P42210, *E*-value = $3e^{-33}$) from *H. vulgare* described by Kervinen et al. (1999). To our knowledge, it is the first report of this kind of protein in maize. Most plant aspartic proteinases occur in seeds and are involved in the processing of storage proteins during ripening and in their degradation during germination (Asakura et al. 2000). Phytepsin was shown to be strongly enhanced by plant tissues undergoing apoptosis (Runeberg-Roos and Saarma 1998). In our experiment, the phytepsin-like protein observed was constitutively expressed, even at the T0 stage, and thus seemed not to be related to apoptosis.

Future studies should attempt to follow transgenic overexpression in the DH229 responsive line to try to observe a decrease in androgenesis response. In the same way, recovery of knockout mutants for this particular gene will be able to achieve the validation of the candidate genes identified in this paper and will be able to contribute to a better understanding of androgenesis in maize.

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